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IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

In re application of

Angela TURIANO

Serial No. 08/817,595

Filed: April 22, 1997

Group Art Unit: 1642

Examiner: G. Bansal

For: PHARMACEUTICAL COMPOSITIONS CONTAINING EXTRACTED MHC MOLECULES, FOR STIMULATION OF THE IMMUNE SYSTEM

TRANSMITTAL OF DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

The attached Declaration was filed (in unexecuted form) with the Amendment filed in the above-identified application on December 5, 2000. The Declaration has now been executed by the inventor and is being filed herewith.

Respectfully submitted,

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IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

In re application of :

Angela TURIANO : Group Art Unit: 1642

Serial No. 08/817,595 : Examiner: G. Bansal

Filed: April 22, 1997 :

For: PHARMACEUTICAL COMPOSITION FOR STIMULATION OF THE IMMUNE SYSTEM

Declaration Under 37 C.F.R.§1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

I, Angela Turiano, being duly warned, declare the following:

1. I am a citizen of Italy, residing at Via Goldoni 51, Milano, Italy, I-20100.

I practiced in the Institute of General Pathology, University of Milan, Italy, from about 1993 to 1996.

2. The following experiments were conducted under my supervision.

3. Experiments were conducted to test *in vivo* effects of MHC preparations obtained from calf liver on mesothelioma cells (in rats), on colon tumor cells (in mice), and on human colon adenocarcinoma cells (in nude rats).

4. A preparation containing MHC molecules (called "AIM"), and a control preparation (called "T-14") which lacks MHC molecules, were obtained from calf liver, using the following protocol.

AIM was obtained from calf liver by the method reported by Wiman et al. (1982). Biochemistry 21, 5351, with minor modifications. Briefly, 30 g of calf liver was chopped and homogenized in a Waring Blendor in the presence of 40 ml of 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl. The treatment was carried out at 11,400 rpm for four periods of 30 sec. each, at intervals of 30 sec. The resulting homogenate was centrifuged at 4°C for 10 min, at 50,000 x g.

The pellet was discarded and the supernatant further centrifuged for 60 min. at 110,000 x g. The pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 50 mM cysteine, 20 mM EDTA to a 20 mg/ml final protein concentration. To 36 ml of the preparation, 100 mg of papain were added and the sample incubated for 60 mn. at 37°C. Iodocetic acid was then added to a 55 mM final concentration, and the pH was adjusted to 8.0 by the addition of 1 M NaOH. The resulting preparation was centrifuged for 60 min. at 160,000 x g and the pellet discarded. The supernatant (35 ml; 2.5 mg protein/ml) was dialyzed extensively against 20 mM Tris-HCl, pH 8.0, and subsequently centrifuged for 10 min. at 10,000 x g to remove the turbidity which appeared during dialysis. The resulting sample had a volume of 45 ml and a protein concentration of 0.84 mg/ml.

Fractionation of membrane proteins was performed as follows. 35 ml of the sample obtained from the previous step were loaded onto a DEAE-Sephacel column (total volume: 3 ml) previously equilibrated with 20 mM Tris-HCl pH 8.0. The column was washed with 40 ml of the same buffer, and eluted with 60 ml of a linear gradient, from 0 to 0.5 M KCl, in the same buffer. 2 ml fractions were collected. A broad protein peak encompassing fractions 4 to 19 was detected.

The fractions which tested positive with MoAb H42A (MHC class II D.B.A. Italia, Segrate) were pooled and concentrated. Further purification of the class II antigens was achieved by affinity chromatography on a column of Sepharose 4B containing class II monoclonal antibodies H42A (MHC class II D.B.A. Italia, Segrate). The desorbed material was eluted with the equilibrating buffer containing 3M $MgCl_2$. The class II antigens were separated by preparative electrophoresis under non-denaturing condition performed in slab gels (14 cm width x 0.15 cm thickness using a LKB 2001 apparatus (Pharmacia LKB, Uppsala, Sweden)).

The procedure described by King and Laemmli was adopted with the following modifications: a) SDS was omitted both in stacking and separating gels; b) a linear gel gradient 12% to 18% (by vol.) was present in the separating gel. Electrophoresis was run at a constant current of 40 mA. Throughout the run, temperature was 15°C. To reveal protein bands, a small amount was run in parallel in another lane and Comassie-stained. Two bands were thus revealed. Since the separation was not carried out in the presence of SDS, migration was not unequivocally related to molecular mass. However, a rough assessment effected by comparison with proteins of known molecular mass permitted an estimate of 30 and 40 kda, respectively, for the two

bands. After the run the gel was cut into 2-mm slices. The protein-containing slices were identified by comparison with the Coomassie-stained lane. Each band was contained in two adjacent slices which were extracted with two 3 ml portions of 50 mm Tris-HCl, pH 8.0, by grinding them together finely in a Pottr homogenizer. The two proteins were combined and extensively dialyzed against 20 mM potassium phosphate, pH 7.0, after removing gel debris by centrifugation. The proteins were then concentrated in Centricon-10 microconcentrators to a final volume of 2 ml. Protein content was 54 and 75 μg/ml for lower and higher molecular mass proteins, respectively. The sample was sterilized by filtration on nitrocellulose filters (pore size: 0.22 nm). This substance was named AIM.

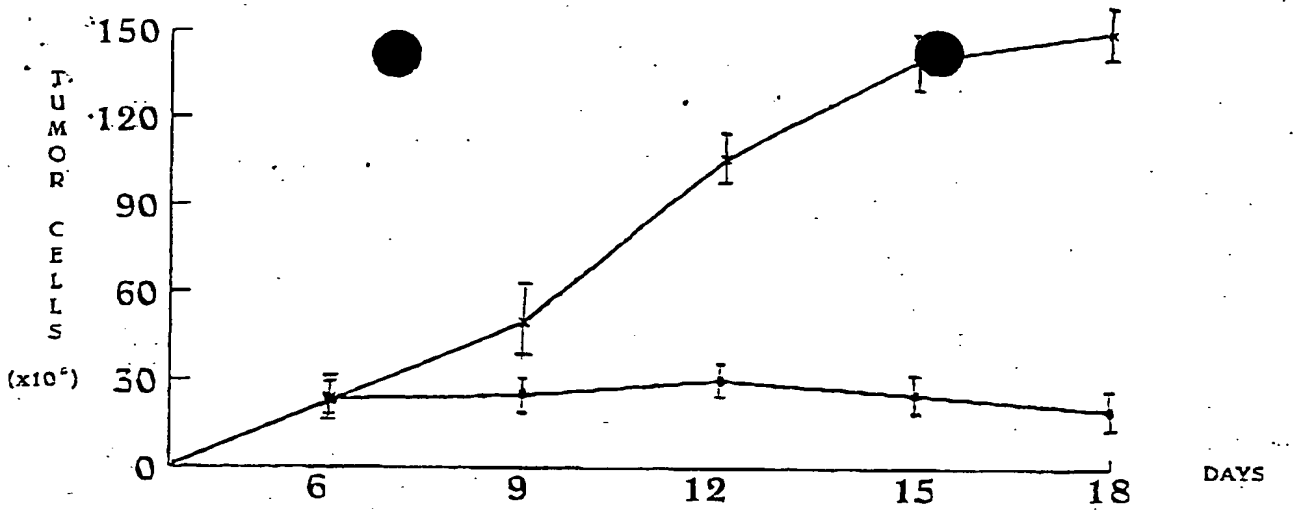
Fractions which tested negative with MoAb H42A and H58A (MHC class I D.B.A. Italia, Segrate) were also pooled, concentrated and named T-14 (data not shown).

5. The "AIM" and "T-14" fractions were tested on rats bearing mesothelioma cells, as follows:

Anti-tumor effects of AIM were investigated using male inbred Fischer rates bearing mesothelioma cells: Two groups of 60 rats were injected with 250,000 mesothelioma cells in the pleural cavity. Tumor cells were counted in both groups 6 days after inoculation. The number of cells was similar in the two groups. Thereafter, 50 rats received AIM (0.5 mg/kg/day) and 50 rats received T-14 in PBS in the same dose as a control. The drug solution was administered from 6 days to 18 days in the pleura of treated rats. Tumor growth was monitored on 9th, 12th, 15th, and 18th day as above.

6. The results with mesothelioma cells are shown below:

The data were subjected to a two-paired t test, and significance was assumed when p was < 0.05. For each experimental point (the mean ± SEM of 10 experiments), bars representing SEM values are shown. When not shown, the bars were smaller than the symbol used. ● represents treatment with AIM, and **x** represents treatment with the T-14 control.



Treatment with AIM clearly reduced mesothelioma cell growth when compared to a control treatment..

7. The "AIM" and "T-14" fractions were tested on mice bearing colon (C26) tumor cells, as follows:

Anti-tumor effects of AIM were investigated using BALBI/c female mice bearing Colon C26 tumor cells: Mice were implanted with 50,000 Colon C26 tumor cells in 0.2 ml of medium RPMI 1640 into the subcutaneous tissue of the dorsal region on day 0. Tumor cells were surgically removed when the diameter of the tumor reached 8 to 10 mm. The mice were divided into two groups and 3 days thereafter, six mice received AIM in PBS (150 μg/mouse) and six received T-14 in PBS at same dose as a control. After 20 days, mice were sacrificed and observed under a stereoscopic microscope to count tumor cells.

8. The results with the colon tumor cells are shown below:

Group	Dose	Mouse	Tumor Recurrence
1	150 μg/ml AIM	No.1	-
		No.2	-
		No.3	-
		No.4	-
		No.5	-
		No.6	-
2	150 μg/ml T-14	No.1	+
		No.2	+
		No.3	+
		No.4	+
		No.5	+
		No.6	+

Treatment with AIM clearly impaired tumor recurrence when compared to a control treatment.

9. The "AIM" and T-14" fractions were tested on nude rats bearing human colon adenocarcinoma cells, as follows:

Nude rats were challenged subcutaneously with $1x10^6$ HT29 human colon adenocarcinoma cells. Starting 8 hours later, they received twice a week 6 local injections of AIM (o.5 mg/kg) or "T-14" at an equivalent dose. Treatment with AIM inhibited growth of HT29 cells in about 50 to 70% of the rats.

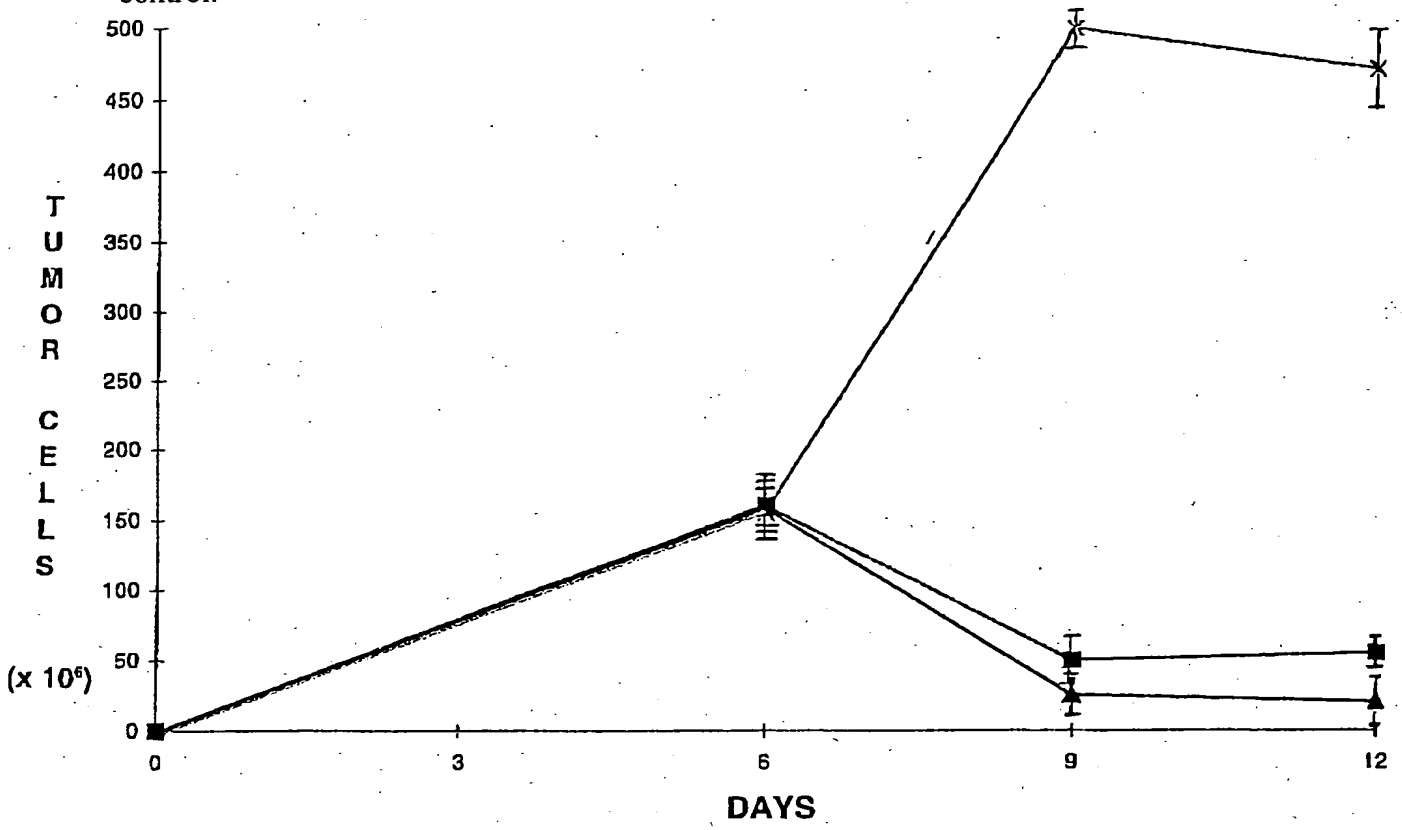
10. The reduction in mesothelioma cell growth in the rats, the impairment of colon tumor cell recurrence in the mice, and the inhibition of human tumor cell growth in the nude rats clearly show that MHC molecules impair cancer cell growth in a cross-species (MHC molecules from calf inhibiting tumor cells from rat, mouse, or human origin) and/or a cross-tissue (liver cell MHC molecules functioning against mesothelioma and colon cells) manner.

11. The following experiment shows that a synthetic peptide from an MHC molecule exhibits antitumor activity which is similar to that of an "AIM" preparation of MHC molecules. This supports the conclusion that the active ingredient in the "AIM" preparation is, in fact, one or more MHC molecules.

Synthetic peptide Kb163-174 (Thr-Cys-Val-Glu-Trp-Leu-Arg-Arg-Tyr-Leu-Lys-Asn), corresponding to an exposed region of the MHC class I molecule, H-$2K^b$, and representing a site of interaction of class I MHC molecules with the corresponding cell receptor (Schneck *et al.* (1989). *Proc Natl Acad Sci USA* 86, 8516-20), was obtained from Sigma (Product No. T8033).

The peptide and the "AIM" preparation were tested for anti-tumor activity as follows: Approximately 250,000 Yoshida AH-130 cells were injected into the pleura of male inbred Fisher rats on day 0; and tumor growth was monitored daily from day 6 to day 12 by counting the number of tumor cells present in the pleural cavity. "AIM," the peptide Kb163-174, or the negative control T-14 as described above, were administered daily from day 6 to day 12 in the pleura of treated rats. The results are shown in the graph below, in which ▲ represents treatment

with AIM; ■ represents treatment with the peptide; and x represents treatment with the T-14 control.



Clearly, both AIM and the peptide significantly reduced tumor growth compared to the control ($p<0.05$) from day 7 onward, to approximately the same extent.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

December 19, 2000
Date

Angela Turiano

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